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Effects of melatonin and N-acetylcysteine on lung ischemia reperfusion injury

Ischemia reperfusion injury

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Abstract
Aim: In our study, we aimed to determine the effects of using melatonin, N-acetyl cysteine, and low potassium dextran solution (LPDS) as a preservative solution on ischemia/reperfusion injury.
Material and Methods: A total of 48 male Sprague-Downey rats were used. Rats were divided into 8 groups: group 1: ischemia, group 2: ischemia+melatonin, group 3: ischemia + N-acetylcysteine, group 4: ischemia+melatonin + N-acetylcysteine, group 5: ischemia + reperfusion, group 6: ischemia + reperfusion + melatonin, group 7; ischemia + reperfusion + N-acetyl cysteine, and group 8; ischemia+reperfusion+melatonin+N-acetyl cysteine. Total antioxidant capacity (TAOC), malondialdehyde (MDA) and neutrophil values of each group were determined. The TAOC was obtained by spectrophotometric measurement of the absorption level of the color formed by the hydroxylion formed by the Fenton reaction with orthodianisidine. The MDA value was obtained spectrophotometrically using the thiobarbituric acid method, and the Neutrophil count was obtained as cell count using Papanicolau Stain stain.
Results: While the highest TAOC values were in groups 4, 2, 3, 1, the lowest TAOC values were in groups 5,7,6,8. The highest MDA values were in groups 5, 7, 6, 1, 8, whereas the lowest MDA were in groups 4, 2, 3 ($p<0.05$). Neutrophil in bronchoalveolar lavage increased in groups 5,7,6,3,2,8,4.
Discussion: In lung ischemia reperfusion, the use of melatonin and/or N-acetyl cysteine, and LPDS decreases plasma MDA levels and increases TAOC values. Additionally, the use of these antioxidants and preservation solutions decreases cell damage immunohistochemically, the cytokines mediating inflammation, the PMNL, and the inflammatory response associated with them.

Keywords
Ischemia; Melatonin, N-acetylcysteine, Reperfusion

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Introduction

Many cells, mediators, receptors, and free oxygen radicals are responsible for ischemia- reperfusion damage to tissues and organs [1, 2]. Neutrophils, complement and other polymorphous leukocytes are effective in endothelial damage, and free oxygen radicals play a role in ischemia reperfusion injury, especially during the reperfusion phase [3, 4]. The main preservation solutions used in lung transplantations are low potassium dextran solution (LPDS), euro-collins (E-C), and wisconsin solution (W-S) (5).

Melatonin is an effective molecule preventing the toxic effects of free radicals. Having antioxidant properties, this molecule can protect the lung against ischemia reperfusion injury [6, 7]. N-acetyl cysteine, a potent antioxidant and anti-inflammatory, has mucolytic properties and protects the lung against ischemia reperfusion injury [8]. LPDS is one of the modified lung preservation solutions and is used in many lung transplant programs [5].

In our study, we aimed to report the results of the use of melatonin, known to protect the lung from ischemia and has antioxidant activity, N-acetyl cysteine, having mucolytic properties, and LPDS, a preservation solution. Additionally, we tried to determine the total antioxidant capacity (TAOC) and serum Malondialdehyde (MDA) levels, indicators in the evaluation of ischemia reperfusion injury.

Material and Methods

After the approval of Dicle University Ethics Committee (approval date/no: 12.03.2008/18), a total of 48 Spraque-Downey male rats were used in the study. All rats were divided into 8 groups.

Group 1 was the ischemia-only group, group 2 was ischemia+melatonin, group 3 was ischemia+N-acetylcysteine, group 4 was ischemia+melatonin+N-acetylcysteine, group 5 was ischemia+reperfusion, group 6 was ischemia+reperfusion+melatonin, group 7 was ischemia+reperfusion+N-acetylcysteine, and group 8 was ischemia+reperfusion+melatonin+N-acetylcysteine group.

Procedures

After sedation with Sodium-Pentobarbital, a 2 cm collar incision was made in the cervical region. A 0.5 cm incision was made in the trachea, and a 5F tracheostomy cannula was inserted and connected to a ventilator.

The incision line was extended from the cervical region below the xiphoid and total sternotomy was performed. By revealing the left hilum of the lung, the left pulmonary artery was cannulated with a 24G angiocath and heparinized. The atraumatic clamp was placed to the left lung hilum to create ischemia.

Group 1 rats were exposed to ischemia for 2 hours by placing an atraumatic clamp on the left lung hilum. Group 2 rats were administered melatonin 3mg/kg i.p. with LPDS (15 min, 15-20cc) 15 minutes before ischemia, and then the hilus was exposed to ischemia for 2 hours. Group 3 rats were given N-acetylcysteine 150mg/kg i.p. with LPDS 15 minutes before ischemia and and then were exposed to ischemia for 2 hours. Group 4 rats were administered melatonin and N-acetylcysteine i.p. with LPDS 15 minutes before ischemia and were exposed to ischemia for 2 hours. Group 5 rats were exposed to ischemia for 2 hours and then reperfused for 2 hours. Group 6 rats were exposed to ischemia for 2 hours, and just before reperfusion, they were given melatonin i.p. with LPDS and were exposed to reperfusion for 2 hours. Group 7 rats were exposed to ischemia for 2 hours, and then N-acetylcysteine i.p. with LPDS was given just before reperfusion, and they were exposed to reperfusion for 2 hours. After being exposed to ischemia for 2 hours, group 8 were given melatonin and N-acetylcysteine i.p. with LPDS just before reperfusion, and the rats were exposed to reperfusion for 2 hours.

At the end of the experiment, approximately 5cc of blood was taken by cardiac puncture for TAOC capacity measurement. Plasma and serum parts were separated, and serum part was kept in deep freezer at -80 C°, and then antioxidant capacity levels were measured in an autoanalyzer (Abbott Aeroroset, USA) with the antioxidant capacity kit developed by Erel. Afterwards, the absorption level of the color formed by the hydroxyl ion formed by the Fenton reaction with orthodianisidine was measured spectrophotometrically in the autoanalyzer. The intensity of the color was evaluated according to the antioxidant capacity, and the test results were calculated as mmol/lt.

At the end of the experiment, the upper lobe of the lung was taken from the rats and washed with isotonic solution. MDA analysis was performed using the thiobarbituric acid method. 0.5gr tissue taken from lung tissue was homogenized with 4.5 ml 5.5% Trichloroacetic acid and kept for 10 minutes at 100°C, then 1ml of thiobarbituric acid was added to the samples that were kept at +4°C for 30 minutes and cooled, and measured spectrophotometrically (Shimadzu, 1800 series, Japan) at 532 nanometers (nmo/gr).

Approximately 1cc of saline was given to the main bronchus of the left lung with 24G Angiocut and aspirated, and the samples were spread on the preparation. The samples were then stained with Papanicolau stain. BAL evaluation was performed at 40 magnification, by counting 50 cells in 2 distant regions where cellularity is most intense. Lymphocytes, Macrophages, PMNL and Resp. Epithelial numbers were recorded. Those with no cells were evaluated as zero. The number of neutrophils was estimated in %.

Tissue samples weighing 1g were taken from the lower lobe of the left lung and sent for pathological examination. Then, all rats were sacrificed by heart puncture.

Statistics

IBM SPSS Statistics 22.0 was used for data analysis. Continuous variables were expressed as mean ± standard deviation, while categorical variables as number-ratio. Homogeneity analysis of variances was done with Levene's test ($p>0.05$). The Shapiro-Wilk test was used to evaluate the normal distribution ($p>0.05$). Results were evaluated with the Kruskal-Wallis test, analysis of variance and the Mann-Whitney-U test. Some results were evaluated by giving different scores to each group among themselves.

Lung alveolar hemorrhage values and parenchymal damage were expressed as scoring. The absence of hemorrhage was scored 0 (grade 0), a single red cell in the alveoli was scored as 1 (grade 1), erythrocyte populations that did not completely fill the alveoli were 2 (grade 2), and erythrocyte populations that completely filled the alveoli were scored as 3 points (grade 3).

In the lung parenchymal injuries, the absence of damage was scored as 0 (normal), focal inflammation as 1 (very mild damage), perivascular, peribronchial edema, vascular congestion and inflammation as 2 (moderate damage), and severe vascular congestion and thrombosis with intrapulmonary-interstitial edema as 3 points (severe damage).

Results

All rats were at the same age, weighing 250-300 grams. The decrease in group 1 TAOC value was found to be statistically significant compared to groups 2, 3, 4 ($p<0.05$), and the decrease in TAOC values in groups 5, 6, 7, 8 was statistically significant compared to groups 1, 2, 3, 4 ($p<0.05$). The increase in group 8 TAOC value was statistically significant compared to groups 6 and 7 ($p<0.05$) (Table 1).

The decrease in MDA value in group 1 was statistically significant compared to groups 2, 3, 4, 5, 6, 7. There was a statistically significant decrease in group 2 MDA value compared to groups 1, 5, 6, 7, 8. The decrease in group 3 MDA value was statistically significant compared to groups 1, 4, 5, 6, 7, 8. The decrease in group 4 MDA value was statistically significant compared to groups 1, 3, 5, 6, 7, 8. On the other hand, the increase in group 5 MDA value was statistically significant compared to all groups. There was a statistically significant increase in group 6 MDA value compared to groups 1, 2, 3, 4, 5. The increase in group 7 MDA value was statistically significant compared to groups 1, 2, 3, 4, 5, 8. Finally, the increase in group 8 MDA value was statistically significant compared to groups 2, 3, 4, 5, 6, 7 ($p<0.05$). While the decrease in MDA values in groups 2, 3, and 4 was found to be statistically significant compared to groups 5, 6, 7, 8, the increase in MDA values in group 5 was statistically significant compared to groups 6, 7, 8. The decrease in MDA values in group 8 was statistically significant compared to group 6, 7 ($p<0.05$) (Table 2). When the TAOC and MDA values of the groups were compared, the increase in the MDA values of groups 5, 6, 7, 8 and the decrease in the TAOC values of the same groups were found to be statistically significant ($p<0.05$). There was a contrast between MDA and TAOC.

According to BAL results, the increase in lavage neutrophil ratio in group 1 was significant compared to groups 2, 3, 4 ($p<0.05$). The neutrophil count of group 1 and 5, which did not receive pharmacological agents, was higher than in the other groups. Considering the groups receiving pharmacological agents, the lowest neutrophil count was in group 8 (Table 3).

When the MDA, TAOC and neutrophil ratios of the groups were evaluated, neutrophil counts of group 8 were statistically low ($p<0.05$). In groups 1 and 5, neutrophil counts in BAL were statistically high, and MDA levels were also statistically high in these groups. On the other hand, TAOC values were found to be low ($p<0.05$) (Figure 1).

When the groups were evaluated in terms of alveolar hemorrhage, all in group 1 were grade 3, one in group 2 was grade 0, 5 in group 2 were grade 1, one in group 3 was grade 0, 4 in group 3 were grade 1, one in group 3 was grade 2, two in group 4 were grade 0, 4 were grade 1, all in group 5 were grade 3, 5 in group 6 were grade 2, one was grade 3, 4 in group 7 were grade 2, 2 were grade 3, 5 in group 8 were grade 2, and one was grade 3 (Figure 2).

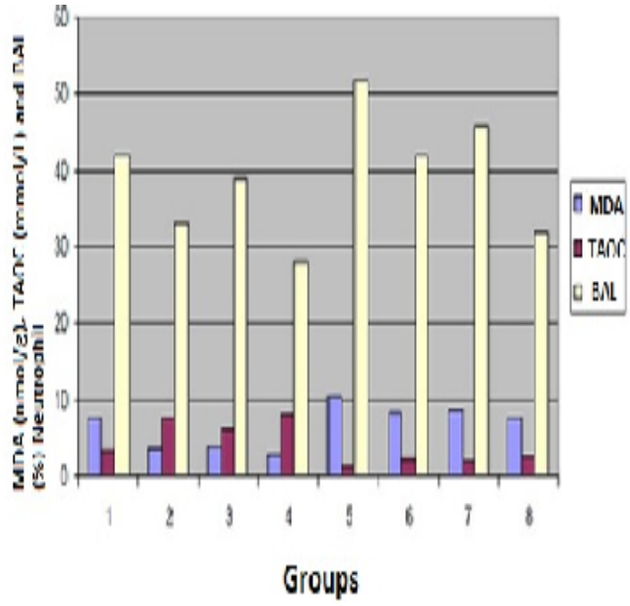

Figure 1. Comparison of MDA, TAOC and Neutrophil (%) ratios

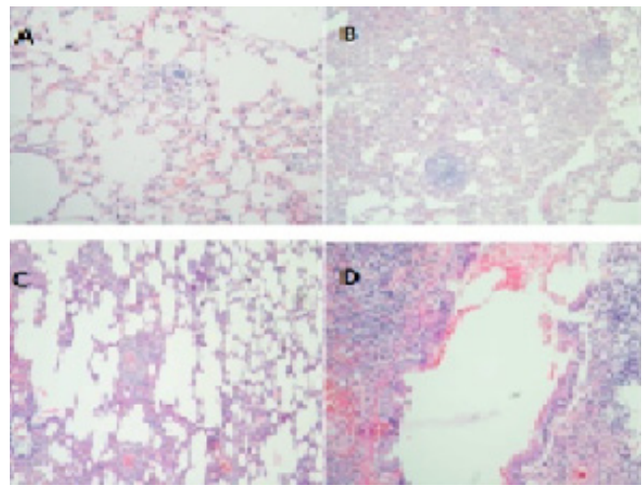

Figure 2. Alveolar Hemorrhage (A; Grade 0), Alveolar Hemorrhage (B; Grade 1), Alveolar Hemorrhage (C; Grade 2), Alveolar Hemorrhage (D; Grade 3)

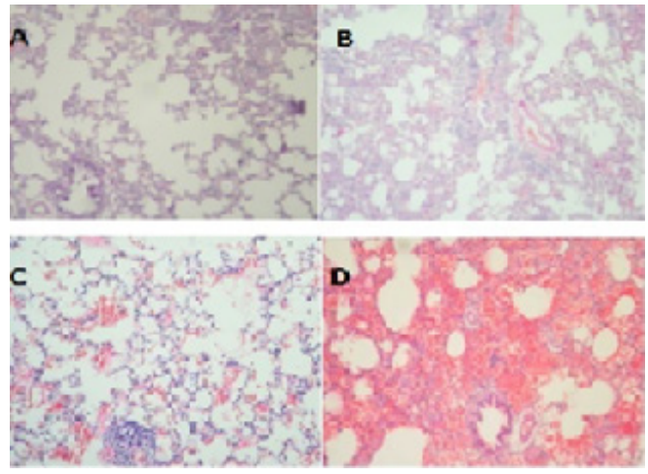

Figure 3. Normal Lung tissue (A), Mild focal inflammation (B), Moderate Perivascular, Peribronchial Congestion and inflammation (C), Severe intrapulmonary-interstitial edema with severe vascular congestion and thrombosis (D)

Table 1. Mean TAOC values and standard deviation of the groups

	Mean ± SD TAOC values (mmol/L)
Group 1 (I)	3,25 ± 1,78
Group 2 (I+M)	7,6 ± 3,12
Group 3 (I+A)	6,28 ± 6,26
Group 4 (I+M+A)	8,3 ± 3,7
Group 5 (I+R)	1,43 ± 1,1
Group 6 (I+R+M)	2,6 ± 1,37
Group 7 (I+R+A)	2,23 ± 1,4
Group 8 (I+R+M+A)	2,76 ± 1,3

I; Ischemia, M; Melatonin, A; N-acetylcysteine, R; Reperfusion, TAOC; Total antioxidant capacity, SD; Standard deviation

Table 2. Mean MDA values and standard deviation of the groups

	Mean MDA ± SD MDA values (mmol/gr)
Group 1 (I)	7,64 ± 0,39
Group 2 (I+M)	3,47 ± 0,51
Group 3 (I+A)	3,89 ± 0,22
Group 4 (I+M+A)	2,97 ± 0,12
Group 5 (I+R)	10,35 ± 1,03
Group 6 (I+R+M)	8,53 ± 0,26
Group 7 (I+R+A)	8,9 ± 0,47
Group 8 (I+R+M+A)	7,6 ± 0,12

I; Ischemia, M; Melatonin, A; N-acetylcysteine, R; Reperfusion, MDA; Malonyldialdehyde, SD; Standard deviation

Table 3. Mean and standard deviation of BAL Neutrophil (%) ratios in the groups

	BAL Neutrophil ratios (%) ± SD
Group 1 (I)	42 ± 2,60
Group 2 (I+M)	33 ± 3,20
Group 3 (I+A)	39 ± 2,15
Group 4 (I+M+A)	28 ± 1,85
Group 5 (I+R)	52 ± 2,94
Group 6 (I+R+M)	42 ± 4,27
Group 7 (I+R+A)	46 ± 3,26
Group 8 (I+R+M+A)	32 ± 3,12

I; Ischemia, M; Melatonin, A; N-acetylcysteine, R; Reperfusion, BAL; Bronchoalveolar lavage, SD; Standard deviation.

When the groups were evaluated in terms of parenchymal damage, all in group 1 had severe, 4 in group 2 had normal, 2 had mild, two in group 3 had normal, 4 had mild, 5 in group 4 had normal, one had mild, all in group 5 had severe, two in group 6 had mild, 4 had moderate, one in group 7 had mild, 5 had moderate, two in group 8 had mild, and 4 had moderate damage (Figure 3).

Discussion

Lung transplantation is the most appropriate treatment method in terminal lung disease. However, graft dysfunction plays an important role in early morbidity and mortality. Graft dysfunction is caused by pathological changes due to ischemia-reperfusion injury, especially in the early period. The most important factor responsible for ischemia reperfusion injury is free oxygen radicals. Melatonin and N-acetylcysteine are also among the most important antioxidants [6-8].

Melatonin is a hormone secreted from the pineal gland with antioxidant and anti-inflammatory effects. N-acetylcysteine is a thiol compound and scavenges free radicals by both nonenzymatic and conjugation and reduction mechanisms. In the initial stage of lung damage due to free radicals, they both penetrate the cell rapidly and act as a scavenger of free radicals [8, 9]. In our study, the effects were evaluated by administering melatonin and N-acetyl cysteine to the subjects, which underwent ischemia/reperfusion. As a result, the antioxidative effects of both melatonin and N-acetylcysteine were shown in accordance with the literature.

Free oxygen radicals are very difficult to show in serum due to their very short lifespan. Therefore, the effectiveness of free oxygen radicals in serum can be evaluated by measuring the serum level of MDA resulting from lipid peroxidation. In many experimental studies, Melatonin has been shown to produce low levels of MDA [7, 10]. In our study, we studied serum levels of MDA to evaluate the effect of free oxygen radicals and detected low MDA levels in the groups given melatonin and N-acetyl cysteine ($p<0.05$).

There are many anti-oxidant molecules available against oxidative damage in the organism. However, their half-life is very short. Therefore, measurement of TAOC is more important. TAOC is the parameter that contributes most to this defense level [11]. In our study, we used Melatonin and/or N-acetyl cysteine to show the efficacy of protecting the lung from ischemia reperfusion injury. The decrease in group 1 TAOC value was found to be statistically significant compared to groups 2, 3, 4 ($p<0.05$). The decrease in TAOC values in groups 5, 6, 7, 8 was statistically significant compared to groups 1, 2, 3, 4 ($p<0.05$). The increase in group 8 TAOC value was statistically significant compared to groups 6, 7 ($p<0.05$).

In their rat studies, Sener et al. found that the antioxidant activity of melatonin and/or N-acetyl cysteine was significantly effective on TAOC and MDA levels [12]. Topal et al. reported in their study that the use of hyperbaric oxygen increased oxidative stress and negatively affected the antioxidant activity in the groups given melatonin [13]. In our study, there was a contrast between MDA and TAOC .

Bronchoalveolar lavage is a method that allows the analysis of proteins, cellular elements and cellular products in the distal air spaces of the lung. In their study, Inci et al. found that BAL nitrite levels were low in the group given melatonin. In another ischemia reperfusion study, BAL neutrophil ratios were low in groups exposed to ischemia and used alpha-2 antagonists, and BAL neutrophil ratios increased in cases with low antioxidant levels [14, 15]. In our study, the increase in lavage neutrophil ratio in group 1 was significant compared to groups 2, 3, 4 ($p<0.05$). The neutrophil count in groups 1 and 5, which did not receive pharmacological agents, was significantly higher. Considering the groups receiving pharmacological agents, the lowest neutrophil count was in group 8.

In lung transplantations, pharmacological preservation solutions and pharmacological drugs are used to prevent

ischemia-reperfusion injury. These are mostly solutions such as E-C, LPDS, W-S. The superiority of LPDS protection solution has been reported in many studies [16, 17].

Kelly et al. investigated whether the preservation solutions changed the membrane potential. In the study, the membrane potential changed in the W-S and E-C given group, the membrane potential did not change in the LPDS given group, LPDS inhibited free reactive oxygen production, and thus prevented organ failure due to primary lung transplantation [18].

Conclusion

The use of melatonin and/or N-acetyl cysteine as well as LPDS as a preservation solution in lung ischemia reperfusion reduces plasma MDA levels and increases TAOC levels. Additionally, the use of these antioxidants and preservation solutions decreases cell damage immunohistochemically, the cytokines that mediate inflammation, the PMNL, and the inflammatory response associated with them. The use of melatonin, N-acetyl cysteine, and LPDS as a preservation solution can be considered a correct choice in ischemia reperfusion situations.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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